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Determination of Fat Soluble Vitamins in Food Matrices Using a Lipase-Catalyzed Reaction Coupled with SFE

Charlotta Turner*[1], Jerry W. King[2] and Lennart Mathiasson[1]
[1]*Department of Analytical Chemistry, Lund University, Box 124, 221 00 Lund, Sweden*
E-mail: charlotta.turner@analykem.lu.se, fax: +46 46 2224544
[2]*Food Quality and Safety Research Unit, NCAUR, ARS, USDA, Peoria, Illinois 61604*

Abstract

An on-line supercritical fluid extraction (SFE)/enzymatic hydrolysis procedure using immobilized lipase has been developed for the determination of fat-soluble vitamins in milk powder. Several lipases were evaluated, of which Novozyme 435 (*Candida antarctica Type B*) showed the highest activity towards retinyl palmitate. SFE parameters such as pressure, temperature, modifiers, flow rate, extraction time and water content were optimized. Retinol and tocopherols were quantitatively analyzed from various food items, such as infant formula, liverpaste and minced meat, using the proposed extraction method and analysis by RP-HPLC with UV and fluorescence detection. The described method is faster and more automated than conventional liquid-liquid extraction, or SFE using off-line saponification, and the results do not differ significantly.

Introduction

Fat-soluble vitamins are important nutrients with key roles in several functions of the human body, such as vision (vitamin A), calcium absorption (vitamin D), antioxidative protection in cell membranes (vitamin E) and blood coagulation (vitamin K)[1]. Lately, there has been notable focus on vitamin E, i.e. tocopherols and tocotrienols, due to their importance as antioxidants, providing protecting against several kinds of cancers, coronary heart disease, Alzheimer's disease, cataract development and arthritis[2]. In order to establish accurate nutritional labeling of food products, it is of major concern to develop fast and robust analysis methods, which also meet the increasing demand of environmental friendliness.

Fat-soluble vitamins are commonly isolated using hot saponification followed by liquid-liquid extraction with hexane and/or ether[3, 4]. Increasing environmental concern on minimizing the use of organic solvents has lead to extensively analytical research during the last decade, which has resulted in several alternative techniques, such as supercritical fluid extraction (SFE)[5, 6]. SFE, even when using a cosolvent consumes only a small quantity of organic solvent. Moreover, SFE is relatively fast compared to classical extraction methods[5], it can be automated, and provides a benign, oxygen-free environment for extracting the heat- and light sensitive vitamins. Applications of analytical SFE for fat-soluble vitamin analysis from food products include carotenoids from vegetables[7], vitamin K_1 from powdered infant formulas[8], vitamin A palmitate from breakfast cereals[9] and vitamin A from calf liver[10]. A method for

determination of fat-soluble vitamins in milk- and meat products utilizing SFE and off-line saponification has recently been developed[11].

In the present study, an alternative to the rather harsh saponification procedure utilized by Berg et al[11] has been investigated. The new approach is on-line enzymatic hydrolysis using an immobilized lipase during the SFE. The aim of the saponification or enzymatically-catalyzed hydrolysis is two-fold; to transform vitamin esters into their parent active vitamins and to provide sample clean-up by converting the coextracted triacylglycerols into lower molecular weight compounds, such as free fatty acids and glycerol.

It has already been shown that certain lipases can sustain high pressures and high temperatures[12], and can be used synthetically in the presence of supercritical media[13]. There are several applications concerning the use of lipases in analytical SFE for total fat determination[14], esterfication of fatty acids into FAMEs for GC analysis[15] and esterfication of milk fat for modification of flavor[16]. In this investigation, lipases have been used to catalyze both hydrolysis and alcoholysis in supercritical carbon dioxide (SC-CO_2). Using these reactions, retinyl esters can be converted into all-trans-retinol, while the reacted acylglycerols produce free fatty acids and fatty acid esters, respectively. Hence, the aim with this study was to find the optimal lipase, immobilized and commercially available, to allow the development of an on-line extraction/enzymatic hydrolysis method for the determination of fat-soluble vitamins A and E in milk powder. The method has then been tested on other food items such as infant formula, liver paste and minced meat.

Experimental procedure

One gram of Novozyme 435 (*Candida antarctica*, Type B) or Lipozyme IM (*Mucor miehei*) (both from Novo Nordisk Inc., Franklinton, NC), or 0.75 gram of Chirazyme L-1 (*Burkholderia cepacia*), L-5 (*Candida antarctica*, Type A) and L-9 (*Mucor miehei*) (from Boehringer Mannheim Corp., Indianapolis, IN), were loaded into the extraction cell at the outlet side. The fresh immobilized lipase filled up one-third to one-half of the total volume of the extraction cell (10-mL). A small layer of Hydromatrix (Varian, Harbor City, CA) was added in order to separate the immobilized enzyme from the sample mixture. One-half gram of sample was weighed accurately and mixed with 1 g of Hydromatrix, and then added on top of the Hydromatrix layer in the extraction cell. Two mL of ethanol (95 %, AAPER Alcohol and Chemical Co., Shelbyville, Kentucky) containing 1-5 % of water (v/v) and 0.1 % of BHT (w/v) (Sigma, St. Louis, MO) was thereafter added on top of the sample mixture in order to optimize the extraction[17]. Samples of infant formula were mixed with the entrainer before

adding 1 g of Hydromatrix, since this kind of matrix is known to be difficult to break[18]. The addition of 1-5% water to the sample with the entrainer has shown to benefit the retinol extraction/reaction, as investigated in an earlier study[19], as well as the fact that some water is needed for the enzymatic hydrolysis[20]. During the entire analytical procedure, care was taken to avoid sample degradation caused by light, oxygen or excessive heat. The samples were extracted with an Isco SFX Model 3560 (Isco Inc., Lincoln, NE), equipped with two 100DX pumps. The extraction fluid used was supercritical carbon dioxide (Air Products, Allentown, PA) modified with 5 vol% ethanol. Collection was performed in 10 mL of ethanol containing 0.1% BHT (w/v). After extraction, the samples were evaporated to dryness under a stream of nitrogen of 99.995% purity (BOC Gases, Murray Hill, NJ) in a Pierce Reacti-Therm heating module (Pierce Chemical Co., Rockford, IL) and then re-dissolved in 1.0 mL of ethanol.

The fat-soluble vitamins were analyzed using an HPLC system consisting of a Waters 600E HPLC pump (Millipore Co., Milford, MA), a Waters 490E variable UV detector, a Thermo Separation FL2000 fluorescence detector (ThermoQuest, San Jose, CA), a Thermo Separation SP8780 autosampler and a ChromQuest chromatography data system (ThermoQuest). Separation was obtained in a Merck column (LiChrospher® RP-18, 5µm, 250 mm length * 4 mm i.d.). A 20 µL injection volume was made into a mobile phase consisting of methanol (Fisher Scientific, Fair Lawn, NJ) and water (98:2/v:v) at a flow rate of 1.0 mL/min. All-trans-retinol and α-tocopheryl-acetate (both from Fluka Chemie AG, Buchs Switzerland) were detected employing a variable UV detector at wavelengths of 325 and 284 nm, respectively. α-Tocopherol (Fluka Chemie AG, Buchs Switzerland), γ- and δ-tocopherol (Sigma, St. Louis, MO) were determined by fluorescence detection using 294 nm for excitation and 330 nm for emission. Retinyl-palmitate (Sigma, St. Louis, MO) was analyzed using the same RP-HPLC system, but with a mobile phase consisting of pure methanol and UV detection at 325 nm. Vitamin contents of the samples were calculated using retention times and peak areas of prepared vitamin standard solutions in a range of 0.1 to 10 µg/mL. Recoveries were based on the average values obtained at 7 laboratories using SFE methodology combined with alkaline saponification (5 of the laboratories) as well as conventional methodology (2 laboratories), which was part of an intercomparison study[21].

Results and discussion

Initial experiments with several commercial available immobilized lipases (Novozyme 435, Lipozyme IM, Chirazyme L-1, L-5 and L-9) together with standard solutions of retinyl-palmitate and α-tocopheryl-acetate in SC-CO_2 showed that Novozyme 435 and Chirazyme L-

5 gave highest recovery of all-trans-retinol. There was no observed enzymatic reaction for α-tocopheryl-acetate with the above enzymes. This is most likely because the active sites of the investigated enzymes are too small to accommodate the bulky aromatic group of α-tocopheryl-acetate. Therefore, recoveries of the alpha isomer of vitamin E in food samples were based on either α-tocopherol alone or the sum of α-tocopherol and α-tocopheryl-acetate. Novozyme 435 was chosen for all further experiments, since it has demonstrated high activity towards triacylglycerols, for quantitative hydrolysis, alcoholysis and interesterfication[22-24]. The entire optimization procedure was thereafter done using samples of 0.5 g milk powder (obtained from AB Västgöta Mjölkförädling, Falköping, Sweden) mixed with 1 g Hydromatrix.

Effects of temperature and pressure. In order to find the optimal extraction/reaction temperature and pressure, two different sets of experiments were performed: One with various temperatures (40, 50, 60, 70 and 80°C) while keeping the density constant at 0.8 g/mL, and another with a range of different pressures (2500 to 7000 psi) with the temperature set to 70°C. These results are shown in Figure 1.

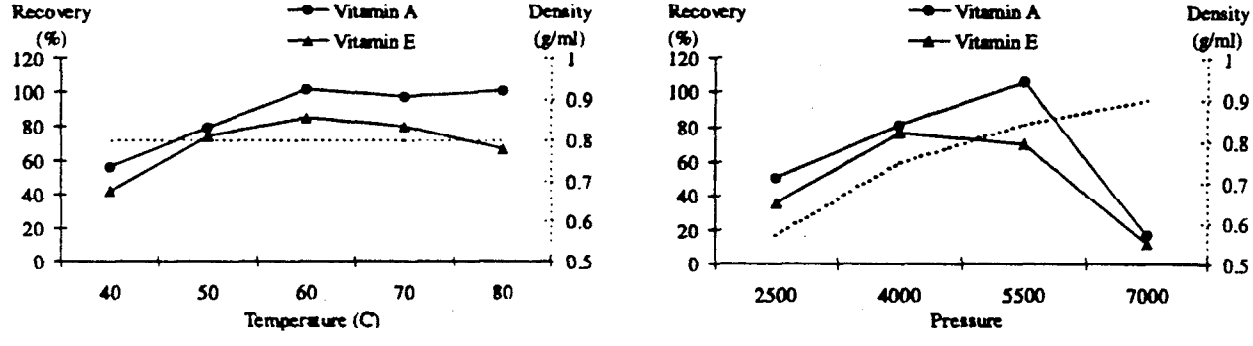

Figure 1 Investigation of effects of temperature (A) and pressure (B) on the recovery of vitamin A and E in milk powder (n=3). The SFE parameters were as follows: A. CO_2 (1 vol% ethanol), 0.8 g/mL (2313-5308 psi for the investigated temperatures 40-80°C), 15 min static and 45 min dynamic extraction at 0.5 mL/min and B. CO_2 (1 vol% ethanol), 70°C, (0.59-0.90 g/mL for the investigated pressures 2500-7000 psi), 15 min static and 30 min dynamic extraction at 0.5 mL/min.

Figure 1.A. implies that the optimal temperature is around 60°C, giving recoveries of 102 and 85% for retinol and α-tocopherol, respectively, whereas a temperature of 80°C seems to cause thermal degradation of α-tocopherol. The retinol recovery is quantitative even at 80°C, which confirms earlier observations of the high thermal stability exhibited by *Candida antarctica* in supercritical CO_2[12]. With respect to the recovery of α-tocopherol as well as the long-term activity of the enzyme, 60°C was chosen as the extraction temperature. The results in Figure 1.B. show that the vitamin recoveries increase with higher extraction/reaction pressure and density, but drop dramatically at 7000 psi, most likely due to inactivation of the enzyme.

Highest recoveries were obtained between 4000 and 5500 psi, where the CO_2 density is 0.77 to 0.85 g/ml. A pressure of 3757 psi was chosen as the optimal pressure, which gives a density of 0.8 g/mL for SC-CO_2 at a temperature of 60°C.

Modifier. A previous study has shown that the addition of 2 mL ethanolic modifier to the sample prior to SFE increases the efficiency of the extraction[17]. In this study experiments clearly demonstrated that the addition of a small amount of water to the entrainer, here 100 µL i.e. 5 vol% of the 2-mL ethanol portion, improved the retinol recovery significantly. This is probably due to that some water is needed for the enzymatic hydrolysis[20]. Moreover, experiments also showed that a delay time of three hours or more between the addition of entrainer to the sample and the starting of the extraction is necessary in order to obtain high recoveries of both vitamins.

Earlier experiments have also shown that 5% modifier is needed to break the milk powder matrix and assure quantitative extraction of the vitamins[17]. However one disadvantage of using high modifier content in combination with enzymatic reactions is that the activity of the enzyme may be adversely affected, thereby diminishing the enzymatic-catalyzed reaction[25]. In order to investigate which one of the effects might be dominating, 1, 2 and 5% of ethanol was added to the carbon dioxide. 2 mL of ethanol containing 5% water was also added to the sample before extraction. The results demonstrated that 5% of modifier gave the highest retinol recovery, i.e. 92%, while 1% ethanol gave 78% and 2% only 81%. This is most likely due to that increasing the modifier content also increases the amount of water in the SC-CO_2. Moreover, the water content of the ethanol probably increases from the time the bottle was opened, i.e. the exact quantity of water that reaches the sample and the enzyme may vary from one set of experiments to another. Anyhow, CO_2/ethanol (95:5/v:v) was chosen as supercritical medium for both extraction/reaction in all further experiments.

Other parameters. Experiments showed that the optimal extraction time in the experiments above was 15 minutes static extraction mode followed by 45 minutes dynamic. The lowest flow rate possible with the Model SFX 3560 instrument, i.e. 0.5 mL/min, gave higher retinol recovery than at 1.0 and 2.0 mL/min, using the same amount of SC-CO_2. The α-tocopherol recovery was not significantly affected by the flow rate, most likely due to the fact that this vitamin is probably not a product of the enzymatic reaction, and does therefore not need a prolonged residence time in the enzyme bed, as is the case for extracted retinyl esters. It was also investigated whether the vitamin recoveries could be enhanced with a different collection solvent and lower collection temperature. In previous studies with the same milk powder, the

highest tocopherol recoveries were obtained using 5°C collection temperature and an ethanol/di-isopropyl ether (1:1) mixture as the collection solvent. In this study however, it was shown that neither a 5°C collection temperature or ethanol/di-isopropyl ether (1:1) improved the tocopherol recovery, relative to the use of pure ethanol at 10°C. This most probably depends on that the coeluting triacylglycerols in the former case now are transformed to more soluble fatty acids and fatty acid esters, which simplifies the collection.

Extraction of various food samples. Milk powder, minced pork- as well as beef meat, low- and high-fat liverpaste and infant formula were analyzed using the developed SFE method. These results are reported in Table 1, as well as results obtained using SFE/off-line saponification and conventional methodologies within the intercomparison study[21].

Table 1 Fat-soluble vitamin content (mg/100 g sample) in various food items using three different extraction methodologies. The SFE parameters for the developed method were SC-CO_2 modified with 5% ethanol, at 60°C and 3757 psi (0.8 g/mL CO_2 density), and 15 minutes static extraction followed by 45 minutes dynamic processing at a flow rate of 0.5 mL/min.

Sample	Vitamin	SFE, On-line hydrolysis /alcoholysis (n=3)	SFE, Off-line saponification[21] (n=3)	Conventional liq-liq extraction[21] (n=3)
Milk powder	all-trans-retinol	0.10	0.11	0.15
	α-tocopherol	0.19	0.42	0.20
Pork meat	all-trans-retinol	0.009	0.004	0.009
	α-tocopherol	0.45	0.42	0.28
Beef meat	all-trans-retinol	0.006	0.005	0.006
	α-tocopherol	0.51	0.62	0.39
Liverpaste (12%)	all-trans-retinol	5.08	5.40	8.77
	α-tocopherol	1.24	1.33	0.36
Liverpaste (23%)	all-trans-retinol	1.32	1.43	2.79
	α-tocopherol	0.26	0.38	0.22
Infant formula	all-trans-retinol	0.37	0.46	0.90
	α-tocopherol	7.84	9.30	7.13
	β/γ-tocopherol	3.33	2.66	5.42

Results in Table 1 obtained using the on-line lipase-catalyzed reaction coupled with SFE do not differ significantly from results achieved with SFE/off-line saponification or conventional alkaline saponification/liquid-liquid extraction. The α-tocopherol recovery from infant formula is based on the sum of extracted α-tocopherol and tocopheryl acetate, since a majority of the alpha isomer in the collected samples was found as untransformed ester. β- And γ-tocopherol occur entirely as parent vitamins, and do not need to be transformed by the lipase. There were no traces of remaining tocopheryl-acetate in any of the other samples. 10% of retinyl-palmitate were found in infant formula and 20-25% in low- and high-fat liverpaste, but were not regarded in the calculation of the all-trans-retinol recoveries shown in Table 1. The reason to the incomplete enzymatic reaction will be further investigated in the near future.

In summary, the developed method is faster than the method using SFE with off-line saponification or conventional extraction techniques, since no additional saponification for sample clean-up is needed. Moreover, it requires less manual operations and only a small amount of organic solvent. The use of the integrated enzymatic hydrolysis/alcoholysis with SFE instead of traditional alkaline saponification provides a more analyte-benign technique for the sensitive vitamin analysis. The investigated lipase *Candida antarctica* catalyzes the transformation of retinyl-esters into all-trans-retinol, which allows direct analysis of vitamin A without any additional hydrolysis step. One disadvantage concerning vitamin E determination is however that if the vitamin occurs as a mixture of tocopherol and tocopheryl esters, also the esters need to be quantified.

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